The Role of β -Hydroxypropionate in Ethylene Biosynthesis[†]

Part II. Ethylene Formation from Propionate-2-¹⁴C in Banana Pulp Slices and Homogenates

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Received March 26, 1970

To elucidate the participation of β -hydroxypropionate in ethylene biosynthesis in banana fruits, ethylene formation in banana pulp slices and homogenates was examined by using propionate-2-¹⁴C as a tracer.

Optimum ethylene formation from propionate-2-¹⁴C was observed in the presence of Mg²⁺, TPP and NADP under an aerobic condition. It was also recognized that ethylene-¹⁴C formation from propionate-2-¹⁴C was diluted by β -hydroxypropionate. β -Hydroxypropionate was proposed as a common precursor for ethylene formation from acetate-2-¹⁴C and from propionate-2-¹⁴C.

As a direct precursor of ethylene biosynthesis in banana fruits, acrylate was postulated.

In a previous report,¹⁾ a possible pathway of ethylene formation from acetate in banana pulp slices is postulated as follows; acetate $\xrightarrow{\text{CO}_2}$ malonate $\rightarrow$ malonic semialdehyde $\rightarrow$ β -hydroxypropionate $\rightarrow$ ethylene.

Spencer and her co-workers^{2~4)} have proposed that β -alanine is a precursor of ethylene in tomato tissues. They postulated acrylate as a direct precursor. Wang *et al.*⁴⁾ suggested a pathway via acrylate by double decarboxylation of fumarate in *Penicillium digitatum*. Although acrylate has been postulated an

immediate precursor for biosynthesis of ethylene, the immediate precursor of acrylate has been thought to be β -hydroxypropionate or fumarate. As shown in the previous report,¹⁾ no incorporation of ¹⁴C from fumarate-2,3-¹⁴C into ethylene in banana pulp slices was found. Therefore, it is expected that β -hydroxypropionate is included in ethylene biosynthesis. If the pathway via β -hydroxypropionate occurs in ethylene biosynthesis of banana pulp slices, propionate may be included as an effective substrate of β -hydroxypropionate biosynthesis. Because, Stumpf *et al.*⁵⁾ reported that propionate was effectively converted to β -hydroxypropionate under an aerobic condition. Moreover, it is well known that there is an oxygen requirement for a step or steps in ethylene biosynthesis. In such a point of view, it is reasonable to consider that propionate is an effective substrate for β -hydroxypropionate biosynthesis.

Therefore, we examined the participation of propionate in the biosynthesis of ethylene by banana pulp slices and homogenates by using propionate-2-¹⁴C. In the present report

[†] This report was presented at the Annual Meeting of the Japanese Society of Plant Physiologists, Kanazawa, April, 1969.

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we show ethylene formation from propionate- 2^{14}C by banana fruit and propose a pathway of ethylene formation from propionate via β -hydroxypropionate.

MATERIALS AND METHODS

The methods used in this study have been described in the previous paper; application of labeled substrates and reagents,¹⁾ preparation of tissue slices,¹⁾ analysis of organic acids,¹⁾ gas chromatography of ethylene¹⁾ and determination of ethylene- ^{14}C and $^{14}\text{CO}_2$.⁵⁾ Propionate- 2^{14}C (10 mCi/mM) was obtained from the Radiochemical Centre, England.

Preparation of homogenates. Banana fruit (*Musa sapientum*) with yellow in their peel color was ground in a mortar with an equal volume of ice cold 0.1 M potassium phosphate buffer, pH 7.0 containing 0.4 M sucrose. The homogenate was then squeezed through two layer of gauze. The resulting filtrate was used as the homogenate after adjusting to pH 7.0 with KOH.

Preparation of β -hydroxypropionate. β -Hydroxypropionate was obtained by alkaline hydrolysis of β -propiolactone, according to Giovanelli *et al.*⁶⁾

Analysis of organic acids. Slice which were administered by propionate- 2^{14}C after a definite time were homogenized with 80% ethanol and centrifuged. The supernatant obtained was divided into cationic, anionic and neutral fractions by using ion exchange resins. Organic acids in the anionic fraction were separated by silica gel column chromatography. Each organic acid was identified by coincidence of their radioactivity and titration values by reagents on the chromatogram. Especially malonate and β -hydroxypropionate were rechromatographed by using a more long column (50 $\times$ 1.5 cm in diameter) and a modified solvent mixture as an elution solvent. Radioactivity was determined by a gas flow counter.

RESULTS

$^{14}\text{CO}_2$ and ethylene- ^{14}C formation from propionate- 2^{14}C

The results in Fig. 1 establishes that the formation of labeled ethylene from propionate- 2^{14}C by the banana pulp. The formation of the gas was affected by gas phases. Under the argon phase ethylene evolution was low and increased by changing it to air. Thus it seems that for the formation of ethylene from propionate air is required.

FIG. 1. Effect of Gas Phase on Ethylene- ^{14}C Formation from Propionate- 2^{14}C by Banana Slices.

Propionate- 2^{14}C (60 μmole , 9×10^5 cpm) was applied onto the surface of two slices (1 $\times$ 1 $\times$ 1.5 cm), and these slices were incubated in a flask under a flow system at 30°C. Argon gas was replaced by air at the point indicated by the arrow.

Incorporation of ^{14}C from propionate- 2^{14}C into organic acids

Figure 2 shows that ^{14}C derived from propionate- 2^{14}C dominantly incorporated into malonate and β -hydroxypropionate, but slightly into the TCA cycle intermediates. This pattern of radioactivity distribution suggests that the oxidation pathway of propionate proposed by Stumpf⁶⁾ and Vagelos⁷⁾ operates

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FIG. 2. Incorporation of ^{14}C from Propionate-2- ^{14}C into Organic Acid Fraction.

Propionate-2- ^{14}C ($0.03\ \mu\text{mole}$, $4.5 \times 10^5\ \text{cpm}$) was applied onto the surface of two slices ($1 \times 1 \times 1.5\ \text{cm}$), and these slices were incubated in the flask under air-flow system for 30 min at 30°C . Solid line; titration, dotted line; radioactivity.

in banana pulp. Thus the occurrence of the main metabolic route of propionate via β -hydroxypropionate can be confirmed in banana pulp.

Effect of β -hydroxypropionate on the formation of ethylene- ^{14}C and $^{14}\text{CO}_2$ from propionate-2- ^{14}C .

If β -hydroxypropionate participates in ethylene formation from propionate-2- ^{14}C , a dilution effect with β -hydroxypropionate in ethylene formation from propionate-2- ^{14}C is expected. Table I indicates that the specific activity of carbon dioxide and ethylene pro-

duced was affected by the addition of β -hydroxypropionate. This indicates that there occurred a dilution effect by β -hydroxypropionate, suggesting that β -hydroxypropionate is an intermediate of ethylene biosynthesis. These results led us to an assumption that β -hydroxypropionate is a common precursor from acetate-2- ^{14}C and from propionate-2- ^{14}C .

Ethylene- ^{14}C and $^{14}\text{CO}_2$ formation from propionate-2- ^{14}C in banana pulp homogenates

Figure 3 shows the formation of $^{14}\text{CO}_2$ and ethylene- ^{14}C from propionate-2- ^{14}C by banana pulp homogenate in the presence of Mg^{2+} , ATP, TPP and NADP. Ethylene- ^{14}C and $^{14}\text{CO}_2$ formation in the homogenates were strikingly reduced compared with that in the slices on fresh weight basis. $^{14}\text{CO}_2$ /propionate-2- ^{14}C (radioactivity) administered and $^{14}\text{C}_2\text{H}_4$ /propionate-2- ^{14}C administered were 1 and 0.07%, respectively, in the pulp slices, and 0.04 and 0.003% in the homogenates. But ethylene- ^{14}C / $^{14}\text{CO}_2$ ratio in the slices and in the homogenates was 7 and 8%, respectively. Also Fig. 3 indicates the presence of a lag phase in $^{14}\text{CO}_2$ production. These results suggest that propionate-2- ^{14}C was oxidized to acetyl-CoA via β -hydroxypropionate, and acetyl CoA entered to the TCA cycle. Therefore, it may be conclusive that ethylene- ^{14}C formation from propionate-2- ^{14}C was not related to the TCA cycle intermediates as a carbon source.

TABLE I. EFFECT OF β -HYDROXYPROPIONATE ON ETHYLENE- ^{14}C AND $^{14}\text{CO}_2$ FORMATION FROM PROPIONATE-2- ^{14}C BY BANANA PULP SLICES

Addition	$^{14}\text{CO}_2$ (cpm)	CO_2 (mg)	Specific activity (cpm/mg)	%	$^{14}\text{C}_2\text{H}_4$ (cpm)	C_2H_4 (m μ l)	Specific activity (cpm/m μ l)	%
None	34700	86	403	100	614	73	8.4	100
β -Hydroxypropionate	9580	74	129	32	56	102	0.5	6

Two slices were rinsed for 10 min in $10^{-3}\ \text{M}$ β -hydroxypropionate. The excess solution was removed with filter paper. The control slices were similarly treated with a 0.1 M potassium phosphate buffer, pH 7.0. Propionate-2- ^{14}C ($0.03\ \mu\text{mole}$, $4.5 \times 10^5\ \text{cpm}$) was applied onto the surface of two slices. These slices were incubated in a Warburg type vessel for 60 min at 30°C in air as gas phase.

FIG. 3. Ethylene- ^{14}C and $^{14}\text{CO}_2$ Formation from Propionate-2- ^{14}C by Banana Pulp Homogenates.

Reaction mixture contained 3 ml of homogenates, 400 μmoles of potassium phosphate buffer (pH 7.0), 100 μmoles of MgSO_4 , 10 μmoles of ATP, 10 μmoles of TPP, 10 μmoles of NADP and 0.1 μmole of propionate-2- ^{14}C (1.5×10^6 cpm) in a total volume of 4 ml. Incubation temp; 30°C . For the preparation of the homogenates, see text. Gas phase: air.

Co-factor requirements in ethylene- ^{14}C and $^{14}\text{CO}_2$ formation from propionate-2- ^{14}C by banana pulp homogenates

Table II shows the requirements of TPP, ATP and NADP for ethylene- ^{14}C formation, and of CoA, ATP and NADP for $^{14}\text{CO}_2$ formation, respectively. Mg^{2+} was not necessary for both of $^{14}\text{CO}_2$ and ethylene- ^{14}C formation. CoA was inhibitory for ethylene- ^{14}C formation. Enhancement of ethylene- ^{14}C formation in the absence of CoA suggests a possible role as a sulfhydryl reagent in the reaction mixture, or the co-factor may have promoted another reaction detrimental to ethylene biosynthesis. On the other hand, decrease of $^{14}\text{CO}_2$ formation in the absence of CoA suggest that propionate enter to the TCA cycle via acetyl CoA after giving rise to acyl intermediates. The co-factor requirement in $^{14}\text{CO}_2$ and ethylene- ^{14}C formation from propionate-2- ^{14}C in the homogenates are quite similar to these in the modified β -oxidation of propionate

TABLE II. CO-FACTOR REQUIREMENTS FOR ETHYLENE- ^{14}C AND $^{14}\text{CO}_2$ FORMATION FROM PROPIONATE-2- ^{14}C BY BANANA PULP HOMOGENATES

Systems	Radioactivity (Ratio) (%)	
	$^{14}\text{CO}_2$	$^{14}\text{C}_2\text{H}_4$
Complete	100	100
minus Mg^{2+}	81	114
minus TPP	91	61
minus CoA	60	124
minus ATP	39	32
minus NADP	46	63
minus Mg^{2+} , TPP, CoA, ATP and NADP	21	17
with the boiled homogenates	—	—

The complete system contained 3 ml of the banana pulp homogenates, 400 μmoles of potassium phosphate buffer (pH 7.0), 0.1 μmole of propionate-2- ^{14}C (1.5×10^6 cpm), 100 μmoles of MgSO_4 , 10 μmoles of ATP, NADP, TPP and 1 μmole of CoA, in a total volume of 4 ml. Reaction time; 60 min, Gas phase; air.

studied by Stumpf *et al.*⁶⁾ The requirement of these co-factors suggests the participation of the oxidative pathway of propionate in ethylene biosynthesis.

Effect of sealing of incubation chamber on ethylene- ^{14}C and $^{14}\text{CO}_2$ formation from acetate-2- ^{14}C and from propionate-2- ^{14}C by banana pulp slices
As recognized in the previous report,¹⁾

TABLE III. EFFECT OF SEALING OF INCUBATION CHAMBER ON ETHYLENE- ^{14}C AND $^{14}\text{CO}_2$ FORMATION FROM ACETATE-2- ^{14}C AND FROM PROPIONATE-2- ^{14}C BY BANANA PULP SLICES

	From acetate-2- ^{14}C ($^{14}\text{C}_2\text{H}_4/^{14}\text{CO}_2$) (%)	From propionate-2- ^{14}C ($^{14}\text{C}_2\text{H}_4/^{14}\text{CO}_2$) (%)
Sealed system	17	6
Open system (Air flow)	2	13

Each value represents the mean of four replications. Incubation time; 60 min. Acetate-2- ^{14}C (60 m μmoles , 1.1×10^6 cpm), propionate-2- ^{14}C (60 m μmoles , 9×10^6 cpm).

ethylene- ^{14}C formation from acetate-2- ^{14}C increased in the sealed chamber. We suggested the presence of the CO_2 -enhanced formation of ethylene- ^{14}C and suggested the participation of malonate (produced by carboxylation acetyl CoA) in ethylene- ^{14}C formation from acetate-2- ^{14}C . If so, such enhancement should not be found in ethylene- ^{14}C formation from propionate-2- ^{14}C . As expected, ethylene- ^{14}C formation from propionate-2- ^{14}C was not increased in the sealed chamber, in contrast to that of from acetate-2- ^{14}C (Table III).

DISCUSSION

Burg and Burg⁸⁾ first reported the formation of ethylene- ^{14}C from propionate-2- and -3- ^{14}C in apple tissue. Lieberman *et al.*⁹⁾ observed an increase of ethylene formation from tomato sections infiltrated with propionic acid. However they showed that propionic acid labeled at C-1, C-2 and C-3 produced no labeled ethylene when supplied to tomato sections. They concluded that the stimulative effect of propionic acid with tomato, therefore, may be indirect. Meheriuk *et al.*³⁾ also observed the enhancement of ethylene formation by propionic acid. But they have not given in their paper further proof on the formation from propionic acid. The present result has demonstrated the formation of ethylene from propionic acid -2- ^{14}C via β -hydroxypropionate in the banana pulp.

The occurrence of propionic acid in free form is rare in plant tissues.¹⁰⁾ According to Ueda *et al.*¹¹⁾ propionic acid extractable with ether after acidification with sulfuric acid was not found in the banana pulp through the

ripening period. However, they found propionic acid after the treatment with a KOH-methanol mixture of the material, and they referred propionic acid found in such a form to bound propionic acid. The content of the bound form strikingly increased at the maximum climacteric stage and after then slightly decreased at the post climacteric stage. Therefore, though in plant cells propionic acid is rare in free state, the contribution of bound propionic acid to ethylene formation is expected.

Ethylene formation by banana pulp homogenates was repressed as compared with that of the intact tissue. It has been well known that ethylene formation system delicately changes for change in turgor, tissue wounding and tissue destruction. Therefore, further improvement to obtain an active-ethylene forming preparation is necessary.

When a substrate for propionate biosynthesis is added to an *in vivo* system, stimulative formation of ethylene may be recognized. For example, Ogata *et al.*¹²⁾ indicate that iso-leucine but not leucine stimulated the formation of ethylene in tomato tissues. Iso-leucine but not leucine is known to form propionyl CoA as an intermediate in the degradation pathway of iso-leucine. Methional is well known as an effective reagent of ethylene formation in an artificial system (Cu^{2+} -ascorbate system,¹³⁾ FMN-light system¹⁴⁾ and peroxidase system.¹⁵⁾ However, more recently it was reported that methionine was more effective than methional as a substrate of ethylene formation *in vivo*.¹⁶⁾ Thus the present author wishes to suggest that exogenous methionine is also an effective precursor of

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propionate biosynthesis and postulate a hypothetical pathway as follow on the basis of the metabolic pathway of methionine.¹⁷⁾

methionine $\rightarrow$ β -keto-butyrates $\rightarrow$ propionate $\rightarrow$ acrylate $\rightarrow$ ethylene

Ethylene formation from valine, iso-leucine and threonine seems to be elucidated with such a metabolic sequence as a propionate forming substrate.¹⁸⁾

It is well known that the hydroxylating reaction of fatty acids,¹⁹⁾ aromatic com-

pounds²⁰⁾ and steroids²¹⁾ requires both oxygen and reduced pyridine nucleotides. Especially it is noticeable that the proposed hypothetical, but possible biosynthetic pathway of acrylic acid resembles to the biosynthesis of unsaturated fatty acids catalysed by a hydroxylating enzyme.¹⁹⁾

As a conclusion of the previous and the present reports, the following route of ethylene biosynthesis is postulated.

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